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Inflammation-induced reactive nitrogen species cause proteasomal degradation of dimeric peroxiredoxin-1 in a mouse macrophage cell line

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Abstract

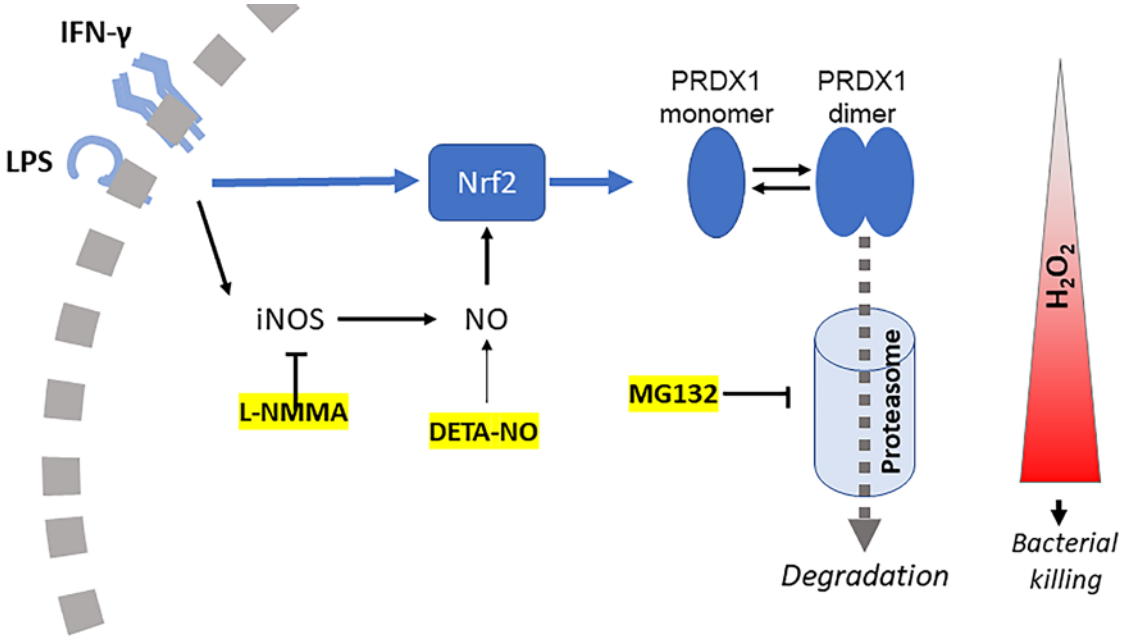
Peroxiredoxin 1 (PRDX1) is an antioxidant enzyme that, when secreted, can act as a proinflammatory signal. Here we studied the regulation of intracellular PRDX1 by lipopolysaccharide (LPS) and interferon-gamma (IFNγ) in the RAW 264.7 mouse macrophage cell line. While LPS or IFNγ alone did not affect PRDX1 protein levels, their combination led to an almost complete loss of the PRDX1 dimer. This was likely mediated by the increased production of nitric oxide (NO) as it was reversed by the NO synthase inhibitor L-N-methylarginine (L-NMMA), while a NO-releasing agent decreased PRDX1 levels. Inhibition of the proteasome with MG132 also prevented the loss of the PRDX1 dimer, suggesting that the decrease is due to a NO-activated proteasomal degradation pathway.

By contrast with the decrease in protein levels, LPS increased PRDX1 mRNA and this effect was amplified by IFNγ. Two other Nrf2 target genes, thioredoxin reductase (TXNRD1) and heme oxygenase (HMOX1), were also induced by LPS but IFNγ did not increase their expression further. This study shows that inflammation differentially regulates PRDX1 at the levels of protein stability and gene expression, and that NO plays a key role in this mechanism.

Keywords

Peroxiredoxin; inflammation; nitric oxide; proteasome; Nrf2

Graphical abstract



Introduction

Peroxiredoxins (PRDX) are highly conserved thioredoxin (TXN) peroxidases [1], reducing peroxides including hydrogen peroxide (H_2O_2) and peroxynitrite, and are one of the most abundant cellular proteins, comprising 0.1-0.8% of the total soluble protein in mammalian cells [2]. Intracellular PRDXs have anti-inflammatory roles as shown by several studies using overexpression or knock-out mice (for instance [3, 4]). On the other hand, extracellular PRDXs can trigger production of inflammatory cytokines by macrophages [5, 6], acting as danger signals, and have been implicated in the initiation of sterile inflammation in cerebral ischemia [7].

We have previously shown that stimulation of macrophages with the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) leads to the release of preformed PRDX1 and PRDX2 [6, 8]. However, only the disulfide-linked homodimers of PRDX1 and 2 are secreted; the monomers are not secreted and cysteine mutants that cannot form dimers are not secreted [8].

Interferon-gamma (IFNγ) is a Th1 cytokine that acts in synergy with LPS to augment TLR4-mediated proinflammatory responses [9] and has an essential role in the innate immune responses against bacteria [10, 11]. A key effect of the combined stimulation with IFNγ and LPS in macrophages is the induction of inducible nitric oxide (NO) synthase (iNOS) and subsequent NO production [12], which also participates in the innate immunity against intracellular pathogens [13, 14].

In this study, we investigated the effect of LPS and IFNγ on the expression of PRDX1 in the RAW 264.7 macrophage cell line, focusing on the oxidized dimer. To investigate the role of NO in the regulation of PRDX1, we measured NO production and used specific inhibitors of iNOS. Finally, because of earlier studies showing that PRDX1 is susceptible to proteasomal degradation following nitrosative stress [15], and the proteasome can be activated by reactive nitrogen species [16], we tested the role of the proteasome using its inhibitor MG132. Our results uncover a novel pathway of NO-dependent, proteasome-dependent regulation of PRDX1 levels that can provide new insights into the mechanisms of redox regulation of inflammation and immunity.

Methods

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, USA) unless otherwise stated. Recombinant rat IFNγ was from R&D Systems, Minneapolis, MN.

Cell culture and treatment

RAW 264.7 cells were maintained at 37°C and 5% CO_2 in RPMI containing 2mM L-glutamine, supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, USA) and 10% (v/v) heat inactivated Foetal Calf Serum. For experiments, $1.25x10^5$ cells/ml were seeded in 24-well plates and incubated overnight to allow adhesion. Stimulations were performed as indicated in the text.

LPS was dissolved in PBS at 200 µg/ml, then diluted in medium to have the final concentration. IFNγ was prepared from a stock solution at $2x10^6$ U/ml in culture medium. LPS was used at 10ng/ml as this study followed up from previous studies from this laboratory where gene expression analysis revealed induction of PRDX1 mRNA at 6h [17]. The IFNγ concentration of 100U/ml was also used in our previous work on the same cells [6]. Macrophage pre-incubation with IFNγ is typically used to increase LPS-induced macrophage activation, in terms of cytokine production and iNOS. In the literature, times of incubation vary from 16h to few hours. We preincubated with IFNγ for 2h based on previous works [18] and, since this pre-treatment increased nitrate production in our experimental model, this scheme was used for all the experiments.

Western blot

Cells were gently washed in PBS which was removed completely before 150µl 1.5% Laemmli sample buffer containing 50 mM N-ethylmaleimide (NEM) (to prevent modification of protein free thiols) was added to each well. After centrifugation at 5000 x G for 5 minutes at 4°C to remove any remaining cells or cellular debris, proteins were analysed by SDS-PAGE (12% acrylamide) under non-reducing conditions, transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK) and probed for PRDX1 or PRDX2 (rabbit polyclonal antibodies were a generous gift from Dr Eva-Maria Hanschmann, University of Greifswald, Germany). Membranes were then incubated with an anti-rabbit HRP-linked secondary antibody (Sigma-Aldrich) at 1:25,000 and developed using ECL

Western Blot Analysis System (GE Healthcare). Blots were stripped with Restore™ Western Blot Stripping buffer (Thermo Fisher Scientific) and reprobed with rabbit monoclonal anti-GAPDH antibodies (Cell Signaling Technology, Danvers, Massachusetts, USA). All primary antibodies were diluted 1:1,000. Densitometry was performed on blots using ImageJ 1.48v.

Samples represent biological replicates, i.e. each sample in the various lanes was an independent cell culture well processed independently.

Measurement of NO production

Nitrite levels were measured by the Griess reaction [19]. The assay was performed on cell supernatants using Griess reagent system (G2930) (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions.

TaqMan RT-qPCR

Total RNA was extracted from cells using QIAzol (QIAGEN, Hilden, Germany), following manufacturer's instructions. Reverse transcription (RT) and TaqMan qPCR analysis of *PRDX1* (Mm01621996_s1), *TXNRD1* (Mm00443675_m1) and *HMOX1* (Mm00516005_m1) gene expression were performed as previously reported [17]. Data were normalised to *HPRT1* (Mm00446968_m1) and expressed as fold change vs one control sample. All TaqMan gene expression assays were purchased from Life Technologies (Carlsbad, USA).

Results

PRDX1 loss occurs following IFN/LPS stimulation

Addition of LPS or IFNγ independently did not affect intracellular levels of PRDX1. In contrast, treatment with IFNγ 2h prior to addition of LPS and further culture for 24 h caused loss of the PRDX1 dimer (Fig. 1A). Either LPS or IFNγ alone did not affect intracellular PRDX1 monomer or dimer, following normalization for protein content using GAPDH as loading control, as shown in the densitometric analysis in Fig. 1B, whereas IFN/LPS markedly decreased the amount of PRDX1 dimer, an effect that was not evident for the monomer. The loss of intracellular PRDX1 dimer was not due to its release in the extracellular environment since no PRDX1 could be detected in the supernatants of IFN/LPS-stimulated

cells (data not shown). Identical results were obtained with the other 2-Cys PRDX, PRDX2, except that no PRDX2 monomer was detected in any of the samples (Supplementary Fig. 1).

To further investigate the decrease in the levels of PRDX1 following exposure to IFN/LPS, a time course was performed over 24h. The results, shown in Fig. 1C, indicate that the decrease in PRDX1 dimer is first observed at 18h, and is significantly reduced by 24h post-stimulation, in the absence of any significant change for the monomer.

Role of NO and proteasomal degradation in the loss of PRDX1 dimer

A key aspect in the synergy between IFNγ and LPS is the upregulation of iNOS [12], therefore the nitrite content of the time course supernatants was investigated as an indicator of NO production. As shown in Fig. 2A, the time-course of nitrite production paralleled that of the decrease in PRDX1 levels (Fig. 1C), peaking at 24h, suggesting a potential link between the two phenomena. Of note, stimulation using either LPS or IFNγ alone did not induce nitrite production (Fig. 2B), paralleling the effect on PRDX1 (Fig. 1B).

Therefore, we tested inhibitors of iNOS in our model, firstly looking at their effects on nitrite production. In these experiments, cells were incubated with 0.3 mM N-G-nitro-L-arginine methyl ester (L-NAME) or L-N-methylarginine (L-NMMA) for 1h prior to the 2h stimulation with IFNγ, and subsequent 24h stimulation with LPS. Nitrite production was then measured in cell culture supernatants. The results shown in Fig. 2C indicate that, in this model, L-NMMA was the most effective iNOS inhibitor to significantly reduce nitrite production. It is possible that L-NAME is less effective than L-NMMA in our model as L-NAME needs to be hydrolysed to its active free acid form by esterases [20].

To investigate the role of iNOS in the PRDX1 decrease in cells treated with IFN/LPS, we tested the effect of 1 mM L-NMMA added 1h prior to IFN/LPS stimulation. As shown in Fig. 3A, L-NMMA limited the IFN/LPS-induced loss of PRDX1 dimer. To further confirm that the observed decrease in the levels of PRDX1 could be attributed to NO production, we investigated the effect of the NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONO) on PRDX1 levels. Of the available NO donors, DETA-NONO was selected due to its long half-life and steady NO

release [21]. As shown in Fig. 3B, DETA-NONO markedly decreased PRDX1 levels. The possible role of the proteasome in the degradation of PRDX1 was investigated using the proteasome inhibitor MG132. Addition of MG132 1h prior to IFN/LPS prevented the loss of PRDX1 dimer induced by IFN/LPS (Fig. 3C).

PRDX1 gene expression is induced by iNOS

In a different set of experiments, we investigated the effect of IFN/LPS on *PRDX1* gene expression by PCR. As shown in Fig. 4A, the combination of IFN/LPS (2h preincubation with IFNγ before addition of LPS and further 6 h culture) increased *PRDX1* mRNA levels to almost double that of LPS alone, which was not expected as the same conditions caused a large reduction of the PRDX1 dimer (Fig. 1). Because *PRDX1* is a Nrf2 target gene, we also measured two other prototypical Nrf2 target genes, *HMOX1* [22] and *TXNRD1* [23]. As shown in Fig. 4, panels B and C, both genes were induced by LPS alone, with IFNγ having no additional effect. To investigate the role of iNOS-generated NO in the observed increase in *PRDX1* mRNA, we treated cells with L-NMMA 1h prior to 18h IFN/LPS stimulation (2h preincubation with IFNγ before addition of LPS and further 18 h culture). As shown in Fig. 4D, IFN/LPS-induced *PRDX1* mRNA was significantly reduced as a result of iNOS inhibition.

Discussion

The results presented in this study describe a mechanism by which IFN/LPS, but not either alone, decrease PRDX1 protein levels in macrophages possibly by an iNOS-activated proteasomal pathway. Furthermore, our results demonstrate that *PRDX1* gene expression is induced by IFN/LPS, an effect that is also, at least in part, mediated by iNOS. Interestingly, previous work by Diet et al. [24], while showing an induction of PRDX1 mRNA by IFN/LPS , did not report any change in total PRDX1 protein levels. There are, however, several differences between our study and that of Diet et al. [24]. First of all we used RAW 264.7 cells instead of bone marrow-derived macrophages [24]. Secondly, the previous study was primarily focused on mRNA expression and the level of PRDX was not specifically investigated (with only one protein lane shown). More importantly, we have used gels run in

non-reducing conditions to distinguish between the PRDX1 monomer and the disulfide-linked dimer because we were particularly interested in the dimer that is the secreted form in macrophages, as we have extensively shown elsewhere [6, 8, 25]. Given that we found that the loss of PRDX1 occurs preferentially at the level of the dimer, measuring total PRDX1, as it would happen with reducing SDS-PAGE where dimers are all reduced to monomers, could mask the effect on the dimer.

The experiments reported here also suggest that the loss of PRDX1 is linked to induction of iNOS which would activate proteasomal degradation as shown by Grune et al. with aconitase [26]. Our hypothesis is also in agreement with a previous study showing that PRDX1 is polyubiquitinated by the ubiquitin protein ligase E6AP, which is activated by nitrosative stress [15].

It should be noted, however, that there are alternative explanations for the decrease of the PRDX dimer, such as overoxidation to an over-oxidized form of the monomer (e.g. sulfonylated or nitrosylated PRDX) that thus cannot form the dimer; it is also possible that an over-oxidized dimer is formed, but this may not be recognized by the antibody with the same efficiency as the fully-reduced monomer. In the case of PRDX2, this is suggested by the microheterogeneity of the monomer in Supplementary Fig. 1. We noted this microheterogeneity before and attributed this to the presence of a glutathionylated dimer [6].

A further limitation of this work was that L-NMMA only inhibited NO production, as detected by extracellular nitrite formation with the Griess' reagent, only by one third while the effect on PRDX stabilization is by over 60%. This may be due to various factors that can interfere with the formation of nitrites, and studies with other techniques for measuring NO more specifically could address the issue.

Our finding that LPS-induced PRDX1 mRNA is increased by IFNγ at least partially through NO supports the hypothesis of a requirement for iNOS-derived NO in the activation of Nrf2, the transcription factor that regulates the transcription of PRDX1 [24, 27, 28]. However, concluding that IFNγ/LPS induces PRDX1 mRNA through iNOS-mediated Nrf2 activation would be an oversimplification. First of all, as shown in Fig. 4, LPS alone increases PRDX1 mRNA without increasing NO (Fig. 2B). Second, the synergy between IFNγ and LPS was not

observed for two other Nrf2 target genes, HMOX1 and TXNRD1, where IFNγ did not have an effect in addition to that of LPS. The fact that NO, or reactive oxygen species (ROS), are not the only mediators of LPS-induced Nrf2 activation has already been demonstrated by Cuadrado et al. [29] who showed that Nrf2 activation by LPS can take place in the absence of its redox-sensitive regulator Keap1. However, given the inhibitory effect of L-NMMA on PRDX1 mRNA induction by IFNγ/LPS, we can conclude that both NO-dependent and independent pathways contribute to the increase in gene expression by IFNγ/LPS.

Further studies will be needed to investigate the biological consequence of the loss of PRDX1 dimer after IFNγ/LPS. It is tempting to frame this observation in the context of innate immunity against intracellular pathogens. The primary function of PRDX1 is to eliminate H_2O_2, and peroxides are a major means by which macrophages kill mycobacteria [30]. The same study showed that mycobacterial PRDX is a major defence mechanism [30]. One could therefore hypothesize that PRDX degradation is part of the mechanisms by which IFNγ and LPS promote oxidative killing of pathogens. The hypothesis outlined in the graphical abstract attempts to put our findings in this context and may help designing future studies to investigate the biological significance of PRDX regulation by cytokines.

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Disclosure of interest

The authors report no conflict of interest.

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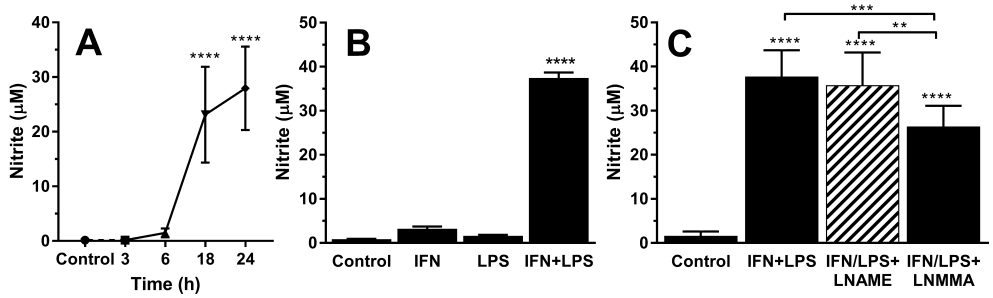


Figure 1. PRDX1 loss following IFN/LPS stimulation.

(A) Representative Western blot analysis of intracellular PRDX1 in control cells (lanes 1-3) or cells treated for 24h with 100U/ml IFNγ (lanes 4-6), 10ng/ml LPS (lanes 7-9) or both (IFN/LPS, lanes 10-12). (B) Densitometric analysis of intracellular PRDX1 dimer (white bars) and monomer (black bars) in cells treated for 24h with IFNγ and LPS, normalised for loading by comparison to GAPDH levels. (C) Intracellular PRDX1 from cells treated with IFNγ and LPS for 6-24h (white bars, dimer; black bars, monomer). Data are mean ± SD of PRDX1/GAPDH densitometric ratio from two independent experiments (B: control, n=5, LPS, n=6; IFN, n=3; IFN/LPS, n=6; C: control, n=4; all other groups, n=6). **P<0.01, *P<0.05 versus respective untreated control by one-way ANOVA and Dunnett's multiple comparisons test.

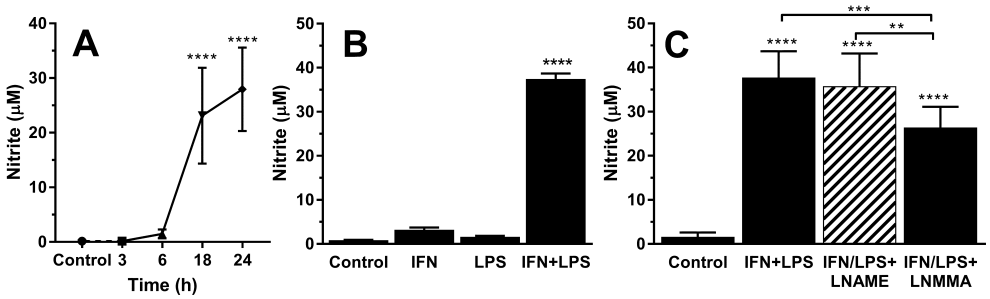


Figure 2. IFN/LPS-induced nitrite production and inhibition by L-NMMA.

Nitrite levels were measured in supernatants from RAW 264.7 cells. (A) Time course of nitrite production by cells stimulated for 2h with 100U/ml IFNγ, then 10ng/ml LPS for the time indicated (*n*=6). (B) Nitrite production from cells stimulated with either IFNγ, LPS or IFN/LPS for 24h (*n*=3). (C) Nitrite production from cells incubated with 0.3mM L-NAME or L-NMMA for 1h prior to IFN/LPS stimulation for 24h (*n*=9). Data are mean ± SD. **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA and Dunnett's (A,B) or Tukey's (C) multiple comparisons test.

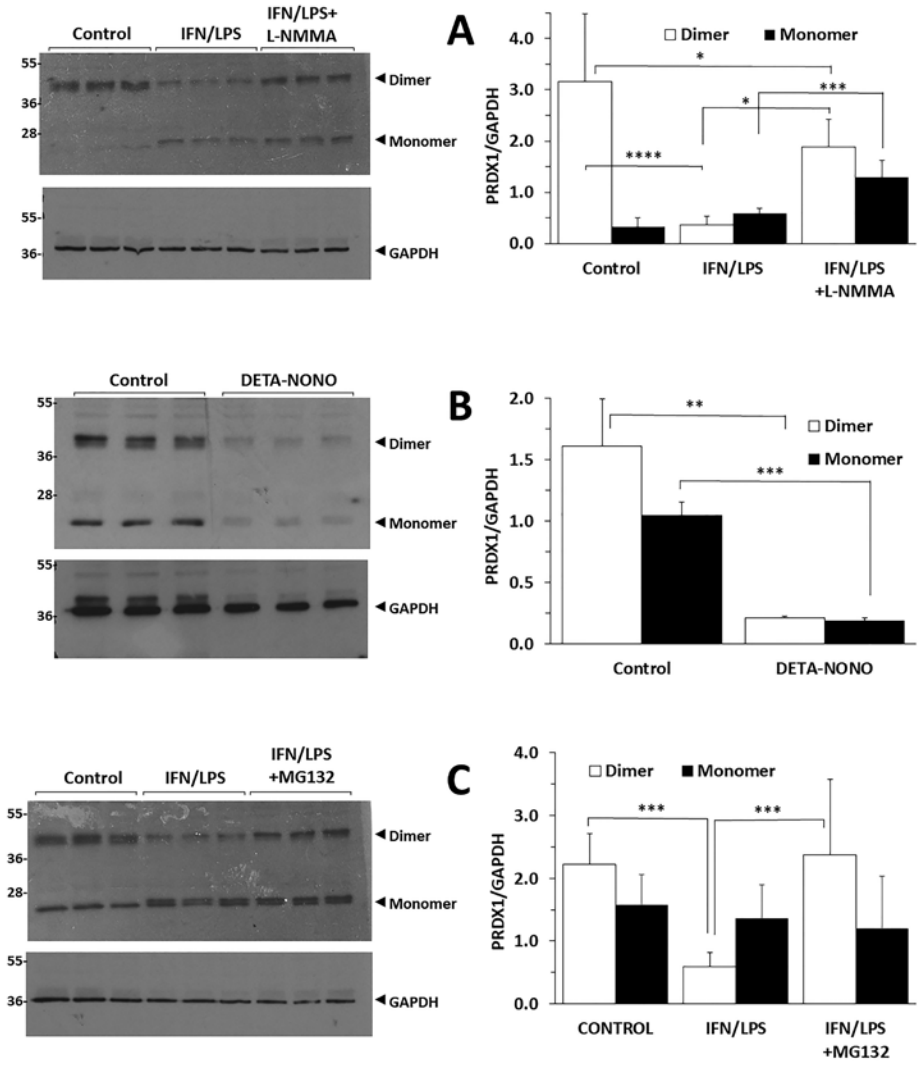
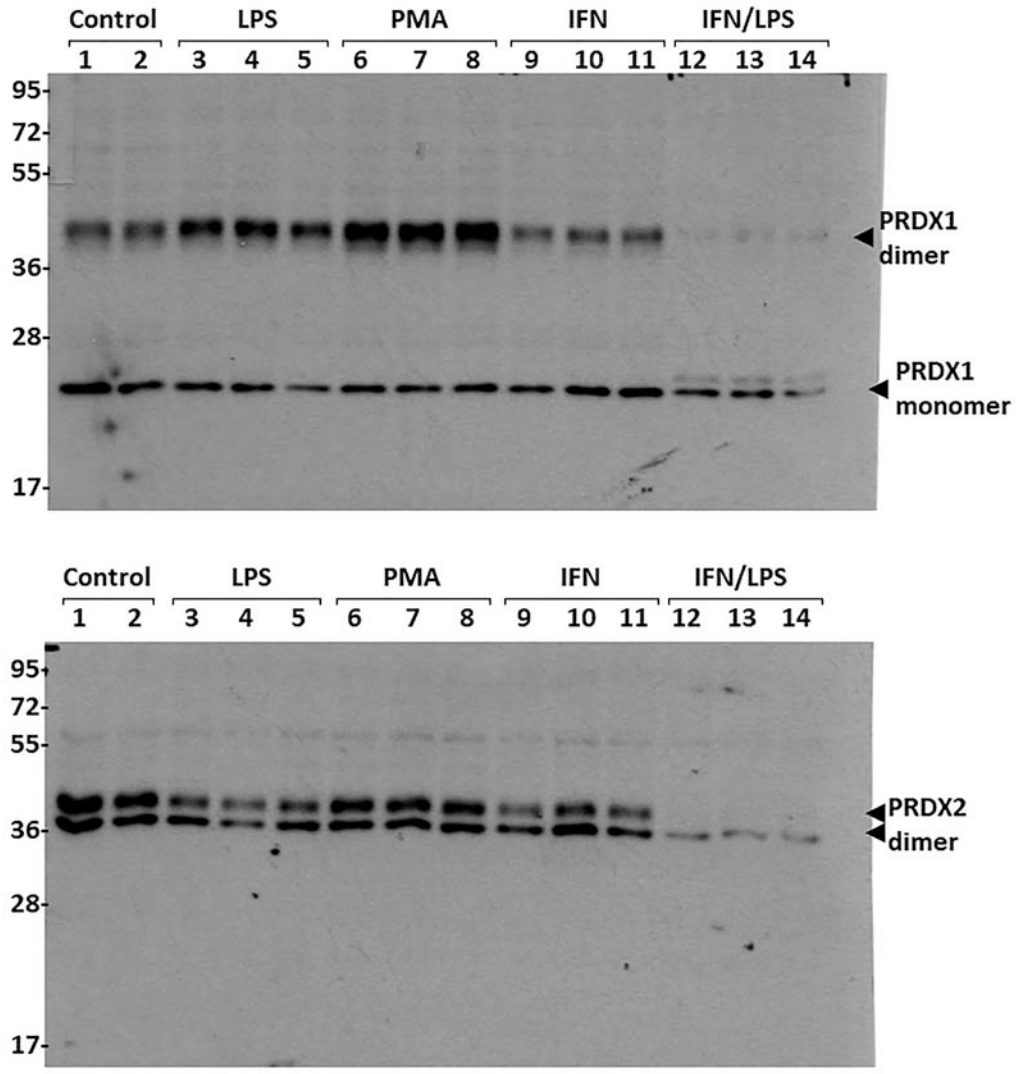


Figure 3. Inhibition of iNOS and proteasome prevent IFN/LPS-induced PRDX1 protein degradation and NO donor induces PRDX1 loss.

Representative Western blot (left) and densitometric analysis (right) of PRDX1 Western blots, normalised for equal loading with GAPDH densitometry. (A) Cells treated for 1h with 1mM L-NMMA, then 2h with 100U/ml IFNγ and 24h with 10ng/ml LPS. (B) Cells treated for 24h with 0.2mM DETA-NONO. (C) Cells incubated for 1h with 0.2µM MG132, then 2h with 100U/ml IFNγ and 24h with 10ng/ml LPS. White bars, PRDX1 dimer; black bars, PRDX1 monomer. Data are mean ± SD of PRDX1/GAPDH densitometric ratio (A, C, n=3-6; B, n=3). A, C: *P<0.05, ***P<0.001, ****P<0.0001 by one-way ANOVA and Tukey's multiple comparisons test. B: **P<0.005, ***P<0.001 vs control by Students t-test.



Supplementary Data

Supplementary Fig. 1. Western blot analysis of intracellular PRDX1 (top) and PRDX2 (bottom) protein from RAW 264.7 control cells (lanes 1-2) or cells treated for 24h with 10ng/ml LPS (lanes 3-5), 500ng/ml PMA (lanes 6-8; PMA is not relevant for the present paper but is present to avoid cutting and pasting the Western blot picture), 100U/ml IFNγ (lanes 9-11) or 10ng/ml LPS after pre-treatment for 2h with 100U/ml IFNγ (lanes 12-14). The two Western blots represent two different gels, not the same membrane stripped and re-probed with a different antibody.